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miR-134 inhibits epithelial to mesenchymal transition by targeting FOXM1 in non-small cell lung cancer cells

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ABSTRACT

Recent studies have implied that miRNAs act as crucial modulators for epithelial-to-mesenchymal transition (EMT). We found that miR-134 expression correlated with invasive potential and EMT phenotype of NSCLC cells. Functional assays demonstrated that miR-134 inhibited EMT in NSCLC cells. In addition, we showed that Forkhead Box M1 (FOXM1) is a direct target of miR-134. Knock-down of FOXM1 reversed EMT resembling that of miR-134 overexpression. We further found that FOXM1 was involved in TGF-β1-induced EMT in A549 cells. These findings suggest that miR-134 acts as a novel EMT suppressor in NSCLC cells.

1. Introduction

Lung cancer is a highly malignant carcinoma, and most deaths of lung cancer are caused by metastasis [1]. Recent researches have demonstrated that epithelial-to-mesenchymal transition (EMT) is a key process contributing to cancer metastasis, characterized by the loss of the epithelial marker E-cadherin, an increase in the mesenchymal markers vimentin and N-cadherin, and an increase in the migratory and invasive behavior [2]. Growth factors, including especially transforming growth factor-β1 (TGF-β1), orchestrate the EMT of various epithelial tissues [3–5]. It is identified as a main inducer of EMT in lung adenocarcinoma and pancreatic cancer [6,7].

MiRNAs are small, non-coding RNAs that modulate gene expression post-transcriptionally. Aberrant expression of miRNAs occurs in many types of cancers, some of which function as tumor suppressor genes or oncogenes [8,9]. Some studies have implied that miRNAs act as crucial modulators for EMT [10–12]. Recently, it was found that the steady state level of miR-134 is modulated by members of the p53/p73/p63 family as part of a miRNA-tumor suppressor network [13]. However, for miR-134, the possible roles and related target genes in non-small cell lung cancer (NSCLC) are still not well elucidated.

In this study, we found that miR-134 expression correlated with invasive potential and EMT phenotype of NSCLC cells. Functional assays demonstrated that miR-134 inhibited EMT in NSCLC cells. Moreover, FOXM1, a potential metastasis promoter, was identified as a direct and functional target of miR-134.

2. Materials and methods

2.1. Cell lines and cell transfection

Four human NSCLC cell lines, including H1395, A549, Calu1 and H1299, were grown in Dulbecco's modified Eagle medium (DMEM) (GIBCO BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS) with 100 μg/ml penicillin/streptomycin at 37 °C with 5% CO_2. For the induction of EMT, cells were treated with 5 ng/ml TGFβ (transforming growth factor β) 1 as described previously [6].

miR-134 mimics and negative control mimics (NC), miR-134 inhibitors (anti-miR-134) and negative control inhibitors (anti-NC) and FOXM1 siRNAs were synthesized by GenePharma Company (Shanghai, China). Transfection was performed with Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's protocol. Total RNA and protein were prepared 48 h after transfection and were used for qRT-PCR or Western blot analysis.

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2.2. Vector construction

Wild-type 3′ untranslated region (3′UTR) of FOXM1 containing predicted miR-134 target sites were amplified by PCR from H1299 cell genomic DNA. Primers used: Forward: GAT CTG CAG TTG CCC CTG TGC TCA AGC TG; Reverse: GAT CAT ATG AGA AAC ATC TAA TAG CGC AC. Mutant 3′UTRs were generated by overlap-extension PCR method. Both wild-type and mutant 3′UTR fragments were subcloned into the pGL3-control vector (Promega, Madison, WI) immediately downstream of the stop codon of the luciferase gene, as described before [14]. DNA fragment coding FOXM1 protein was amplified by PCR from H1299 cell cDNA, and cloned into pCMV-Myc expression vector (Clonetech, Mountain View, CA). Primers used: Forward: GCT GAA TTC GGA TGA AAA CTA GCC CCC GTC GGC; Reverse: CTG CTC GAG CTA CTG TAG CTC AGG AAT AAA C.

2.3. RNA extraction and qRT-PCR

Total RNA was extracted from the cultured cells using Trizol Reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. qRT-PCR was used to confirm the expression levels of miRNAs as described before [15]. U6 snRNA levels were used for normalization.

2.4. Western blot analysis

Protein extracts were prepared by a modified RIPA buffer with 0.5% sodium dodecyl sulfate (SDS) in the presence of proteinase inhibitor cocktail (Complete mini, Roche, Indianapolis, IN, USA). Polyacrylamide gel electrophoresis, tank-based transfer to Immobilon Hybond-C membranes (Amersham Biosciences) and immunodetection were performed with standard techniques. Antibodies against FOXM1 (Novus Biologicals, CO., USA), β-actin (Beijing Zhongshan Biotechnology, Beijing, China), E-cadherin and vimentin (Santa Cruz, CA) were used in western analysis in accordance with the manufacturer's instruction. Signals were visualized with SuperSignal® West Pico chemoluminescent substrate (Pierce, Rockford, III, USA) by exposure to films.

2.5. Luciferase assay

H1299 cells were transfected in 24-well plates using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The transfection mixtures contained 100 ng of firefly luciferase reporter plasmid and 5 pmol of miR-134. pRL-TK (Promega, Madison, WI) was also transfected as a normalisation control. Cells were collected 48 h after transfection, and luciferase activity was measured using a dual-luciferase reporter assay system (Promega, Madison, WI).

2.6. Cell invasion assay

For the invasion assays, 2×10^5 cells were added into the upper chamber of the insert precoated with Matrigel (ECM gel, Sigma–Aldrich, St. Louis, MO). Cells were plated in medium without serum, and medium containing 10% fetal bovine serum in the lower chamber served as chemoattractant. After several hours of incubation, the cells that did not invade through the pores were carefully wiped out with cotton wool. Then the inserts were fixed with 95% ethanol, stained with 0.2% crystal violet, and counted (five high-power fields/chamber) using an inverted microscope.

2.7. Statistical analysis

All data from three independent experiments were expressed as mean ± S.D. Differences were assessed by two-tailed Student's *t* test using Excel software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. miR-134 expression correlates with invasive potential and EMT phenotype of NSCLC cells

We have screened four different NSCLC cell lines (A549, H1395, Calu1 and H1299), for their invasive capabilities and expression of miR-134. We found that miR-134 expression was inversely correlated with invasive capabilities (Fig. 1A and B). Consistent with this, low invasive cells expressed higher endogenous E-cadherin while relatively higher levels of vimentin expression were observed in highly invasive cell lines (Fig. 1B and C). These results suggest that downregulation of miR-134 may contribute to EMT-derived invasive phenotype in NSCLC cells.

3.2. miR-134 regulates EMT in NSCLC cells

Since A549 cells showed significantly higher miR-134 expression (Fig. 1A) compared to H1299 cells, we selected these two cell lines to verify our hypothesis. As shown in Fig. 2A, ectopic transfection of miR-134 mimics led to a dramatic decrease in vimentin expression and a significant increase in E-cadherin expression in H1299 cells. When the A549 cells were treated with anti-miR-134, a decrease in E-cadherin expression and an increase in

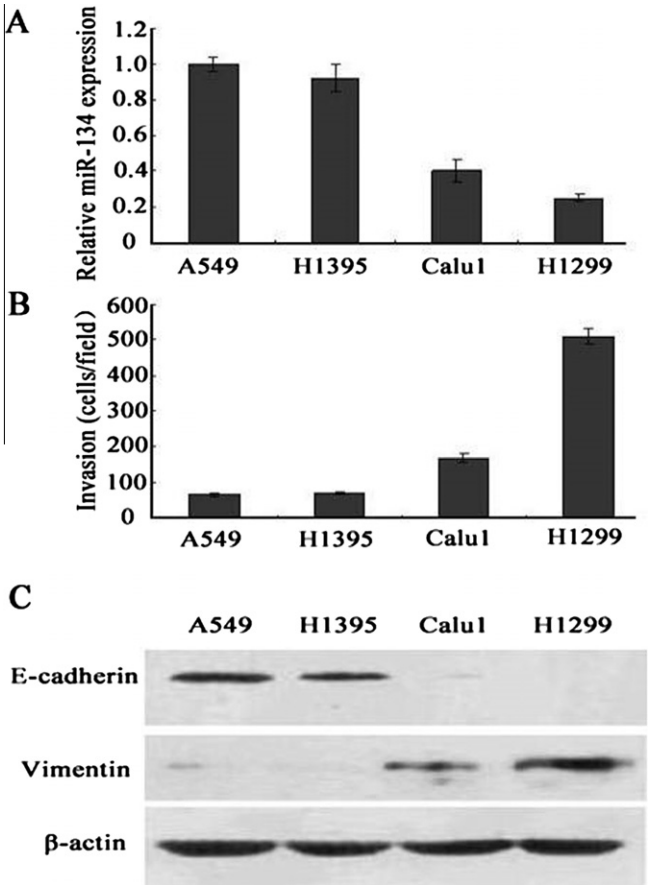

Fig. 1. Association between miR-134 expression and invasive potential and EMT features of NSCLC cell lines. (A) miR-134 levels in the panel of NSCLC cell lines were determined by qRT-PCR. (B) Invasive ability of NSCLC cells was evaluated by invasion assays. (C) Western blot analysis of epithelial marker E-cadherin and mesenchymal marker vimentin in the panel of NSCLC cell lines.

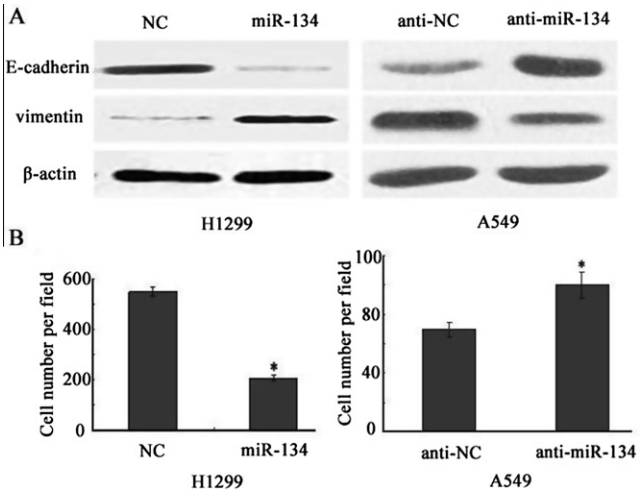

Fig. 2. miR-134 regulates EMT in NSCLC cells. (A) Ectopic expression of miR-134 notably inhibited cell EMT of H1299 cells. Inversely, miR-134 inhibitor stimulated cell EMT of A549 cells. (B) miR-134 inhibit invasion of H1299 cells, while inhibition of miR-134 enhances invasion in A549 cells (*$P < 0.05$).

vimentin expression were observed (Fig. 2A). Furthermore, we found that the increased miR-134 level in H1299 resulted in reduced cell invasion and the reduced miR-134 level in A549 led to enhanced cell invasion (Fig 2B).

3.3. miR-134 directly targets FOXM1

Using online miRNA target prediction databases (miRNA.org and Targetscan), we hypothesized that FOXM1 was a target of miR-134 (Fig. 3A). As shown in Fig. 1A and Fig. 3B, A549 cells showed significantly higher miR-143 expression and lower FOXM1 protein expression compared to H1299 cells. Western blot showed that the enhanced miR-134 in H1299 cells significantly repressed FOXM1 protein expression compared to cells transfected with negative control (Fig. 3C). Relatively, downregulation of miR-134 by inhibitors in A549 cells led to a moderate increase of FOXM1 protein level (Fig. 3C). To further investigate if the predicted binding site of miR-134 to 3′UTR of FOXM1 is responsible for this regulation, we cloned the 3′UTR of FOXM1 downstream to a luciferase reporter gene (wt-FOXM1), its mutant version (mut-FOXM1) by the binding site mutagenesis was also constructed. We co-transfected wt-FOXM1 vector and miR-134 mimics or negative control into A549 cells. The luciferase activity of miR-134 transfected cells was significantly reduced compared to negative control cells (Fig. 3D). Moreover, miR-134-mediated repression of luciferase activity was abolished by the mutant putative binding site (Fig. 3D). These results indicate that miR-134 downregulates FOXM1 expression by directly targeting its 3′UTR.

3.4. FOXM1 is involved in TGF-β1-induced EMT in A549 cells

To fully explore the roles of FOXM1 in EMT, we treated A549 cells with TGF-β1. As illustrated in Fig. 4A, si-FOXM1 reversed the expression of E-cadherin and vimentin induced by TGF-β1 treatment, and seemed to change the morphology from mesenchymal-like spindle-cell shape to epithelial-like appearance in A549 cells, which resembled the inhibitory effects of miR-134. Moreover, TGF-β1 treatment also led to a dramatic increase in FOXM1 expression and a significant decrease in miR-134 expression (Fig. 4B and C). In addition, utilizing pCMV-Myc expression vector, FOXM1 construct without wild 3′UTR was generated to carry out rescue experiments. As shown in Fig. 4D, the inhibition effect of miR-134 on EMT was rescued when FOXM1 expression vector was cotransfected. These results indicated that miR-134 inhibits EMT through regulating FOXM1 expression in NSCLC cells.

4. Discussion

The current work shows that miR-134 expression correlates with invasive potential and EMT phenotype of NSCLC cells. It is also shown that FOXM1 is suppressed by miR-134 via direct binding to the FOXM1 3′UTR. Moreover, we show that FOXM1 is involved in TGF-β1-induced EMT in A549 cells. The date from this study suggest that miR-134 acts as a novel EMT suppressor in NSCLC cells.

EMT is the key process driving cancer metastasis [2,16]. Loss of E-cadherin expression and gain of vimentin expression are considered to be the most important molecular markers of EMT [17,18]. Recent studies have implied that miRNAs act as crucial modulators

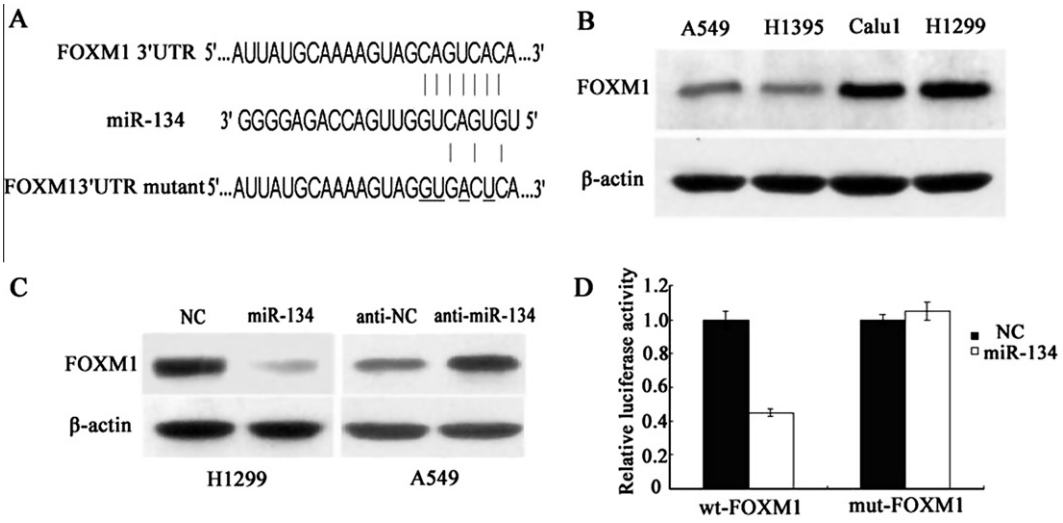

Fig. 3. miR-134 directly targets FOXM1 by binding to its 3′UTR. (A) The predicted miR-134 binding site within FOXM1 3′UTR and its mutated version by site mutagenesis are as shown. (B) Variable FOXM1 expression in four NSCLC cell lines was obtained by Western blot. (C) In comparison with negative controls, miR-134 inhibited FOXM1 protein expression, while reduction of miR-134 by inhibitors moderately restored FOXM1 expression. (D) Luciferase assays indicated that miR-134 downregulated the expression of FOXM1 by binding with its 3′UTR.

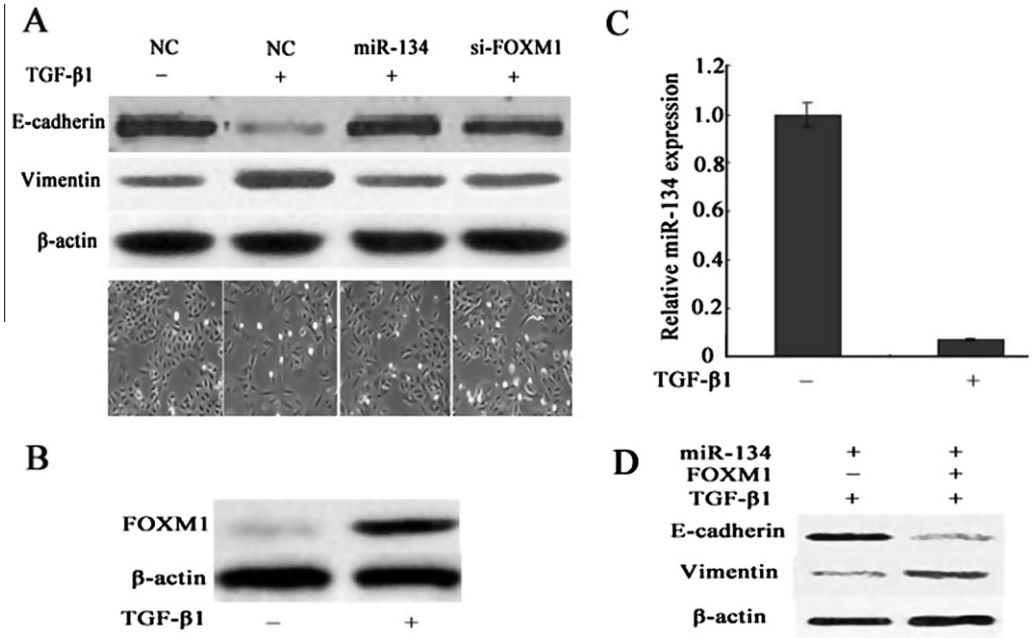

Fig. 4. FOXM1 is involved in TGF-β1-induced EMT in A549 cells. A549 cells were transfected with NC, si-FOXM1 or miR-134 respectively, after 24 h, TGF-β1 (5 ng/ml) was added, and cells were further incubated for 48 h. (A) Western blot analysis of epithelial marker E-cadherin and mesenchymal marker vimentin. At the same time, A549 cells morphological changes were examined. The effect of TGF-β1 on FOXM1 (B) and miR-134 expression (C) were examined. (D) The inhibition effect of miR-134 on EMT was rescued when FOXM1 expression vector was cotransfected.

for EMT. For example, members of the miR-200 family have been shown to inhibit EMT by targeting ZEB1 and SIP1 [10], Moreover, miR-194 inhibits EMT of endometrial cancer cells and miR-30a inhibits EMT in NSCLC [11,12]. Our results show that miR-134 reverses EMT in NSCLC cells. In addition, FOXM1 is involved in TGF-β1-induced EMT in A549 cells.

FOXM1 belongs to a large family of forkhead box (Fox) transcription factors. Overexpression of FOXM1 has been found in a variety of aggressive human carcinomas including lung cancer [19–23]. Interestingly, several studies on FOXM1 implicated its involvement in the early steps of metastasis. For example, FOXM1 was shown to stimulate invasion and angiogenesis of pancreatic cancer cells through induction of matrix metalloproteinase genes MMP-2 and MMP-9, as well as vascular endothelial growth factor (VEGF) [24,25]. Moreover, overexpression of FOXM1 coincides with metastasis of prostate cancer [26]. Our results showed that knockdown of FOXM1 by siRNA or miR-134 reversed EMT in NSCLC cells. These findings imply that dysregulation of FOXM1 by miRNAs may be an important mechanism underlying cancer metastasis.

miR-134 gene is located at 14q32, and is involved in several physiological and pathological processes. For example, miR-134 plays an important role in translation-dependent guidance of nerve growth cones [27]; miR-134 is regarded as a potential plasma biomarker for the diagnosis of acute pulmonary embolism [28]. Interestingly, recent studies indicated that miR-134 may also be involved in carcinogenesis. p53/p63/p73, the tumor suppressors, were believed to be regulators of the miR-134 processing complex [13]. Moreover, Increased expression of miR-134 contribute to the activity of testosterone and $1,25(OH)_2D_3$ to suppress tumor growth in prostate cancer [29]. Our results suggest that miR-134 functions as a potent tumor suppressor in NSCLC.

In conclusion, we newly described miR-134/FOXM1 link and provided a potential mechanism for FOXM1 dysregulation and contribution to NSCLC cell EMT. As a result, restoration of miR-134 expression could have an important implication for the clinical management of NSCLC.

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References

[1] Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., Smigal, C. and Thun, M.J. (2006) Cancer statistics, 2006. CA Cancer J. Clin. 56, 106–130.
[2] Kraljevic Pavelic, S., Sedic, M., Bosnjak, H., Spaventi, S. and Pavelic, K. (2011) Metastasis: new perspectives on an old problem. Mol. Cancer 10, 22.
[3] Kasai, H., Allen, J.T., Mason, R.M., Kamimura, T. and Zhang, Z. (2005) TGF-beta1 induces human alveolar epithelial to mesenchymal cell transition (EMT). Respir. Res. 6, 56.
[4] Cho, H.J. and Yoo, J. (2007) Rho activation is required for transforming growth factor-beta-induced epithelial-mesenchymal transition in lens epithelial cells. Cell Biol. Int. 31, 1225–1230.
[5] Moustakas, A., Pardali, K., Gaal, A. and Heldin, C.H. (2002) Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. Immunol. Lett. 82, 85–91.
[6] Zhang, H.J., Wang, H.Y., Zhang, H.T. and Su, J.M. (2011) Transforming growth factor-beta1 promotes lung adenocarcinoma invasion and metastasis by epithelial-to-mesenchymal transition. Mol. Cell Biochem. 355, 309–314.
[7] Ellenrieder, V., Hendler, S.F., Boeck, W., Seufferlein, T., et al. (2001) Transforming growth factor beta1 treatment leads to an epithelial–mesenchymal transdifferentiation of pancreatic cancer cells requiring extracellular signal-regulated kinase 2 activation. Cancer Res. 61, 4222–4228.
[8] Calin, G.A. and Croce, C.M. (2006) MicroRNA signatures in human cancers. Nat. Rev. Cancer 6, 857–866.
[9] Kumar, M.S., Lu, J., Mercer, K.L., Golub, T.R. and Jacks, T. (2007) Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nat. Genet. 39, 673–677.
[10] Gregory, P.A., Bert, A.G., Paterson, E.L. and Barry, S.C. (2008) The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat. Cell Biol. 10, 593–601.
[11] Dong, P., Kaneuchi, M. and Watari, H. (2011) MicroRNA-194 inhibits epithelial to mesenchymal transition of endometrial cancer cells by targeting oncogene BMI-1. Mol. Cancer 10, 99.
[12] Kumarswamy, R., Mudduluru, G., Ceppi, P., et al. (2012) MicroRNA-30a inhibits epithelial-to-mesenchymal transition by targeting Snai1 and is downregulated in non-small cell lung cancer. Int. J. Cancer 130, 2044–2053.
[13] Boominathan, L. (2010) The tumor suppressors p53, p63, and p73 are regulators of microRNA processing complex. PLoS One 5, e10615.
[14] Liu, Q., Fu, H., Sun, F., et al. (2008) miR-16 family induces cell cycle arrest by regulating multiple cell cycle genes. Nucleic Acids Res. 36, 5391–5404.

[15] Fu, H.J., Zhu, J., Yang, M., Zhang, Z.Y., Tie, Y., et al. (2006) A novel method to monitor the expression of microRNAs. Mol. Biotechnol. 32, 197–204.
[16] Gavert, N. and Ben-Ze'ev, A. (2008) Epithelial–mesenchymal transition and the invasive potential of tumors. Trends Mol. Med. 14, 199–209.
[17] Lee, J.M., Dedhar, S., Kalluri, R. and Thompson, E.W. (2006) The epithelial-mesenchymal transition: new insights in signaling, development, and disease. J. Cell Biol. 172, 973–981.
[18] Grunert, S., Jechlinger, M. and Beug, H. (2003) Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis. Nat. Rev. Mol. Cell Biol. 4, 657–665.
[19] Pilarsky, C., Wenzig, M., Specht, T., Saeger, H.D., et al. (2004) Identification and validation of commonly overexpressed genes in solid tumors by comparison of microarray data. Neoplasia 6, 744–750.
[20] Bektas, N., Haaf, A., Veeck, J., Wild, P.J., et al. (2008) Tight correlation between expression of the Forkhead transcription factor FOXM1 and HER2 in human breast cancer. BMC Cancer 8, 42–50.
[21] Katoh, M. and Human, F.O.X. (2004) Gene family (Review). Int. J. Oncol. 25, 1495–1500.
[22] Wang, Z., Ahmad, A., Li, Y., et al. (2010) Forkhead box M1 transcription factor: a novel target for cancer therapy. Cancer Treat. Rev. 36, 151–156.
[23] Kalin, T.V., Wang, I.C., Ackerson, T.J., et al. (2006) Increased levels of the FOXM1 transcription factor accelerate development and progression of prostate carcinomas in both TRAMP and LADY transgenic mice. Cancer Res. 66, 1712–1720.
[24] Wang, Z., Banerjee, S., Kong, D., Li, Y. and Sarkar, F.H. (2007) Down-regulation of Forkhead Box M1 transcription factor leads to the inhibition of invasion and angiogenesis of pancreatic cancer cells. Cancer Res. 67, 8293–8300.
[25] Dai, B., Kang, S.H., Gong, W., Liu, M., Aldape, K.D., Sawaya, R., et al. (2007) Aberrant FOXM1B expression increases matrix metalloproteinase-2 transcription and enhances the invasion of glioma cells. Oncogene 26, 6212–6219.
[26] Chandran, U.R., Ma, C., Dhir, R., Bisceglia, M., et al. (2007) Gene expression profiles of prostate cancer reveal involvement of multiple molecular pathways in the metastatic process. BMC Cancer 7, 64–85.
[27] Han, L., Wen, Z., Lynn, R.C., Baudet, M.L., et al. (2011) Regulation of chemotropic guidance of nerve growth cones by microRNA. Mol. Brain 4, 40.
[28] Xiao, J., Jing, Z.C., Ellinor, P.T., Liang, D., et al. (2011) MicroRNA-134 as a potential plasma biomarker for the diagnosis of acute pulmonary embolism. J. Transl. Med. 9, 159.
[29] Wang, W.L., Chatterjee, N., Chittur, S.V., Welsh, J. and Tenniswood, M.P. (2011) Effects of 1α,25 dihydroxyvitamin D3 and testosterone on miRNA and mRNA expression in LNCaP cells. Mol. Cancer 10, 58.